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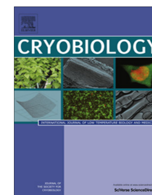

Osteogenesis of cryopreserved osteogenic matrix cell sheets ☆

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ABSTRACT

Cryopreservation of tissue engineered bone (TEB), whilst maintaining its osteogenic ability, is imperative for large-scale clinical application. We previously reported a novel cell transplantation method, in which bone-marrow-derived mesenchymal stem cells (BMSCs) were cultured to confluence and differentiated down the osteogenic lineage to form osteogenic matrix cell sheets (OMCS). OMCS have high alkaline phosphatase (ALP) activity and osteocalcin (OC) contents and can be easily used for producing TEB. The aim of the present study was to investigate whether TEB produced by cryopreserved OMCS maintains sufficient osteogenic potential *in vivo*. OMCS were prepared and divided into three groups according to storage period of cryopreservation (fresh (no cryopreservation), 4 week and 12 week cryopreservation groups). OMCS were cryopreserved by storage in freezing medium (Cell Banker 1®) at −80 °C. Cryopreserved OMCSs were rapidly thawed at room temperature and wrapped around Hydroxyapatite (HA) scaffolds prior to implantation into subcutaneous sites in rats, to determine their *in vivo* bone-forming capability. The constructs were harvested 4 weeks after transplantation and examined histologically and biochemically. Histological analysis of the constructs showed extensive bone formation in the HA pores with high ALP activity and OC content detected in the cryopreservation groups. The present study clearly indicates that cryopreserved/thawed OMCS are still capable of producing mineralized matrix on scaffolds, resulting in bone formation. This cryopreservation technique could be applied for hard tissue reconstruction to ease the cell preparation method prior to time of use.

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Introduction

Bone marrow-derived mesenchymal stem cells (BMSCs) have been widely used for bone tissue regeneration. With rapid advancements in tissue engineering, skeletal diseases including arthritis, bone tumors and osteonecrosis have been successfully treated using tissue-engineered bone (TEB) combined with BMSCs [7,21,28]. At present, preparation of TEB with BMSCs requires several weeks for cultivation to permit BMSC expansion and colonization into scaffolds such as hydroxyapatite (HA) [1,11,17,20,22,23]. In many cases, prolonged TEB preparation times are likely to affect surgery planning owing to timing difficulties. Even planned elective surgeries are often postponed because of patient health, which in some cases makes pre-prepared TEB unusable. Controlling the timing for creation of TEB is therefore of critical importance for its broad clinical use.

As cryopreservation technology has advanced [4,27], cryopreserved cells are expected to become a promising cell source in regenerative medicine. Successful cryopreservation of BMSCs prior to implantation is well documented both preclinically [13,30,32] and in the clinic [10,15]. In fact, cryopreservation and thawing processes have been reported to result in minor effects on both proliferation and differentiation of BMSCs compared with that of non-cryopreserved BMCSs [10,15,27]. Despite these reports, it is still uncommon to create TEB using cryopreserved BMSCs. One reason is that BMSCs are unable to differentiate spontaneously into osteoblasts when cultured in basic culture medium *in vitro* [15,31]. Therefore, cryopreserved BMSCs require additional factors during cultivation after thawing [15].

We previously developed a novel cell transplantation technique for bone formation using BMSCs in the absence of a scaffold [2,3]. BMSCs are cultured in medium containing dexamethasone (Dex) and ascorbic acid phosphate (AscP), and then lifted as single cell sheets (designated as osteogenic matrix cell sheets: OMCSs) with a high osteogenic potential. OMCSs combined with HA result in extensive bone formation *in vivo* [2,3], supporting the use of this

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method for hard tissue reconstruction. This study aimed to determine whether cryopreserved OMCSs combined with HA constructs maintain a sufficient osteogenic potential after *in vivo* transplantation, which may significantly reduce TEB preparation times in the clinic.

Materials and methods

Bone marrow cell preparation

The method of bone marrow cell preparation has been reported previously [2,3,20,22]. Briefly, bone marrow cells were obtained from the femur shafts of 7-week-old male Fischer 344 rats. Both ends of the femur were removed from the epiphysis, and the bone marrow was flushed out using 10 ml of standard culture medium expelled from a syringe through a 21 G needle. Standard culture medium consisted of minimal essential medium (Nacalai Tesque, Kyoto, Japan) containing 15% fetal bovine serum (JRH Bioscience Inc., Lenexa, KS, USA) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Nacalai Tesque).

Harvested cells were transferred into two T-75 flasks (BD Falcon; BD Biosciences, Franklin Lakes, NJ, USA) containing 15 ml of standard culture medium. Cell cultures were maintained in a 95% humidified atmosphere with 5% CO_2 at 37 °C. After reaching confluence, cultured cells were released from the culture substratum using trypsin/EDTA (Gibco, Invitrogen, Carlsbad, CA, USA).

Cell sheet preparation and cryopreservation

The cell sheet preparation method has been reported previously [2,3]. Briefly, BMSCs released using trypsin/EDTA were seeded at 1×10^4 cells/cm^2 in 10-cm dishes (100 × 20 mm; BD Falcon) for subculture in standard culture medium containing 10 nM Dex (Sigma, St. Louis, MO, USA) and 82 μg/ml AscP (L-ascorbic acid phosphate magnesium salt n-hydrate; Wako Pure Chemical Industries, Kyoto, Japan) until confluent OMCSs were formed (approximately day 14). Cells were rinsed twice with phosphate-buffered saline (PBS; Gibco), and then OMCSs were lifted using a scraper. OMCSs were divided into three groups according to the storage period of cryopreservation: fresh, 4-week cryopreservation (4-week) and 12-week cryopreservation (12-week) groups. For cryopreservation, OMCSs were picked up using tweezers, and then placed into cryovials (One OMCS/tube, 2 ml cryogenic vial; BD Falcon) containing 500 μl cryopreservation medium (Cell Banker 1®; Juji Field, Inc., Tokyo, Japan). The cryopreservation medium was sourced commercially; therefore, the cryoprotective agent (CPA) concentration and content are not disclosed, except for inclusion of dimethyl sulfoxide (DMSO).

Tubes were then transferred to a freezer (−80 °C) without programmed freezing steps and stored at −80 °C for 4 or 12 weeks. Cryopreserved OMCSs were rapidly thawed at room temperature, and then rinsed twice with PBS prior to subsequent experimentation. Temperature changes of the cryopreservation medium in cryovials were measured with a thermometer sensor (CENTER 370 RTD thermometer; Center Technology Group, New Taipei, Taiwan). The sensor was inserted into the cryopreservation medium through a hole in the cryovial cap. Temperature changes were recorded during cryopreservation and thawing processes.

Histological examination

Following an additional 24 h of culture in 10-cm dishes containing 10 ml standard culture medium, three OMCSs were fixed in 10% neutral buffered formalin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 2 days, and then embedded in paraffin and cut parallel down the middle prior to staining with hematoxylin and eosin (H&E) for light microscopic observation.

Cell viability assay

To investigate the viability of OMCSs before and after freezing, a method based on tetrazolium reductase activity (Cell Counting Kit-8®; WST-8, Dojindo, Kumamoto, Japan) was employed [8]. Briefly, OMCSs cultured in 6-, 12-, 24- and 48-well plates (BD Falcon; $n = 6$ per plate) were used to generate a standard. The differently sized OMCSs were harvested with a scraper, and then incubated in a 95% humidified atmosphere with 5% CO_2 at 37 °C for 24 h. The samples were then placed in WST-8 solution (100 μl in 1 ml of culture medium) in culture wells. After 3 h of incubation, the solution obtained from each culture well was analyzed by a spectrophotometer (450 nm). Based on the standardization, a linear relationship was obtained between the averaged optical density and seeded cell number per volume of culture medium (cell/ml) (correlation $R^2 = 0.9751$). OMCSs cultured in 6-well plates (BD Falcon; $n = 6$ per plate) were also cryopreserved using the same protocol for 4-week group and 12-week group. Using this standard, the number of viable cells of OMCSs in each group was analyzed before freezing and after thawing. The measurement of cell viability was the same as that applied for the standardization. For all specimens, the cell viability of samples was calculated as a percentage compared with fresh group at 24 h after initiation of the thawing process.

Attachment and survival of cells within the OMCSs was also assessed ($n = 1$ per group) using light microscopy and confocal laser microscopy (Zeiss LSM Axiovert 200; Carl Zeiss, Jena, Germany). Following an additional 24 h of culture in 10-cm dishes containing 10 ml standard culture medium, viable cells were stained with green fluorescent Calcein AM dye and dead cells with red fluorescent Ethidium homodimer-1 dye (LIVE/DEAD® Viability/Cytotoxicity Assay Kit; Invitrogen) following the manufacturer's protocol.

Implantation of HA constructs combined with cell sheets

Porous HA ceramics (50% average void volume, 5 mm diameter by 2 mm thick, Cellyard HA scaffold; Pentax Co, Tokyo, Japan) were used for implantation. Both solid and porous components of the scaffold microstructure were interconnected. OMCSs from each group (fresh, 4- and 12-week groups) were combined with the HA ceramics just after scraping off or thawing to prepare the HA/OMCS constructs. Control HA constructs without OMCSs were also included in the experiment. Each group included six constructs that were implanted subcutaneously into the back of recipient rats ($n = 6$ HA disks per rat) to assess the osteogenic potential of cryopreserved OMCSs.

Radiographic and histological analysis of harvested constructs

At 4 weeks after implantation, all samples were harvested to evaluate osteogenesis. Two disks from each group were fixed in 10% neutral buffered formalin for 2 days, and then analyzed radiographically. After X-ray images were obtained, the samples were decalcified with K-CX solution (Falma Co., Tokyo, Japan), embedded in paraffin and cut parallel down the middle prior to staining with H&E. The remaining disks were stored at −80 °C until measurement of alkaline phosphatase (ALP) activity and osteocalcin (OC) content.

Biochemical analysis

Assays for ALP activity and OC content were carried out according to a method described previously [20]. Briefly, each HA disk

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